#16
Attach

Neal L. First

EDUCATION:

Michigan State College	1948-1952	B.S.	Animal Husbandry
Michigan State University	1954-1957	M.S.	Animal Science
Michigan State University	1957-1959	Ph.D.	Animal Science and Physiology of Reproduction

Ph.D. Thesis Title: Fertility of Frozen Ram Semen

PROFESSIONAL EXPERIENCE:

1956-60	Instructor, Michigan State University-East Lansing.
1960-64	Assistant Professor, Department of Meat and Animal Science, University of Wisconsin-Madison.
1964-1968	Associate Professor, Department of Meat and Animal Science, University of Wisconsin-Madison.
1968-present	Member, Endocrinology-Reproductive Physiology Program, University of Wisconsin.
1968-present	Professor, Department of Meat and Animal Science, University of Wisconsin-Madison.
1987-1990	Joint Appointment in Dept. of Obstetrics and Gynecology, Medical School.
1990	Director USDA-CSRS-ARS National Animal Genome Mapping Program.

HONORS AND AWARDS:

Outstanding Teacher, University of Wisconsin, College of Agricultural and Life Sciences, 1968; Animal Physiology and Endocrinology Award, American Society of Animal Science, 1977; Outstanding Teacher, University of Wisconsin-Madison, 1978; Saddle and Sirloin Club Honorary Recognition Award, 1983; National Association of Animal Breeders National Research Award, 1986; Alexander von Humboldt Award, 1987; University of Wisconsin Distinguished Professor Chair, L.E. Casida Professor of Reproductive Biology and Biotechnology, 1989; Elected to National Academy of Science, 1989; SSR Research Award, 1991, ASAS Morrison Award, 1993.

NATIONAL COMMITTEES:

Acting Director, National Program in Mapping the Genome of Domestic Animals, 1992; National Academy of Science, Institute of Laboratory Animal Research, 1991-1993; NAS Institute of Medicine Committee on Fetal Research and Application, 1992-1993; NAS Class Membership Committee, 1992-1993; National Advisory Board on Ethics in Reproduction, 1994.

SELECTED PUBLICATIONS

Jones, J., Susko-Parrish, J., Navara, C., and **First, N.L.** 1995. **Transcriptional repression in early bovine embryos: effects of overexpression on cdc25 and analysis of RNA polymerase II activation and localization**. Dev. Biol. (in press).

Jones, M.J., and **First, N.L.** 1995. **Aphidicolin treatment induces transcription in 2 cell bovine embryos.** J. Reprod. Fert. (submitted).

Schoff, P.K. and **N.L. First.** 1995. **Manipulation of bovine sperm metabolism and motility using anoxia and phosphodiesterase inhibitors.** Cell Motility and the Cytoskeleton 31:140- 146.

Jones, J.M. and **N.L. First.** 1995. **Expression of cell cycle control protein cdc25 in cleavage stage bovine embryos**. Zygote. 3: May, 1995.

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Susko-Parrish, J., Leibfried-Rutledge, M.L., Northey, D., Schutzkus, V. and **First, N.L.** 1994. Inhibition of protein kinases after an induced calcium transient causes transition of

bovine oocytes to embryonic cycles without meiotic completion. Developmental Biology. 166: 729-739.

Rosenkrans, C.F. and **First, N.L.** 1994. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J. Anim. Sci. 72: 434-437.

First, N.L., Sims, M.M., Park, S.P., and Kent-First, M.J. 1994. Systems for production of calves from cultured bovine embryonic cells. Reprod. Fertil. and Dev. 6: 553-562.

Sims, M.M. and **First, N.L.** 1994. **Production of calves by transfer of nuclei from cultured inner cell mass cells.** Proc. Natl. Acad. Sci. 90: 6143-6147.

Navara, C.S., **First, N.L.** and G. Schatten. 1994. Microtubule organization in the cow during fertilization, polyspermy, paerthenogenesis and nuclear transfer: The role of the sperm aster. Dev. Biol. 162:29-40.

Sims, M.M., and **First, N.L.** 1994. Production of calves by transfer of nuclei from cultured inner cell mass cells. Proc. Natl. Acad. Sci. 90:6143-6147.

First, N.L., and Leibfried-Rutledge M.L. 1993. Nuclear Transfer in Mammals. In: "In vitro fertilization and Embryo Transfer in Primates" (D.P. Wolf, Stouffer R.L., and Brenner, R.M., Eds.), Springer-Verlag New York, Inc. 317-330.

Fulka Jr., J., Leibfried-Rutledge M.L, and **First, N.L.** 1993. Control of germinal vesicle breakdown in bovine x murine hybrid oocytes. Reprod. Nutr. Dev. 33:411-417.

Kim, T., M.L. Leibfried-Rutledge and **First, N.L.** 1993. Gene transfer in bovine oocytes using replication-defective retroviral vectors packaged with Gibbon ape leukemia virus envelopes. Molec. Reprod. Dev. 35:105-113.

Kim, T., M.L. Leibfried-Rutledge and **First, N.L.** 1993. Gene transfer in bovine oocytes using replication-defective retroviral vectors packaged with Gibbon ape leukemia virus envelopes. Molec. Reprod. Dev. 35:105-113.

Prather, R.S. and **First, N.L.** 1993. Cell-to-cell coupling in early-stage bovine embryo: a preliminary report. Theriogenology 39:561-567.

Takahashi, Y. and **First, N.L.** 1993. In vitro fertilization of bovine oocytes in the presence of theophylline. Anim. Reprod. Sci. 34:1-8.

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Rosenkrans, Jr., C.F., G.Q. Zeng, G.T. McNamara, P.K. Schoff and **First, N.L.** 1993. Development of bovine embryos in vitro as affected by energy substrates. Biol. Reprod. 49:459-462.

Rosenkrans, Jr., CF., and **First, N.L.** 1993. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J. Anim. Sci. 72:434-437.

Yom, H.C., R.D. Bremel and **First, N.L.** 1993. Mouse mammary tumor virus promoter directs high-level expression of bovine μS1 casein in the milk of transgenic heterozygous and homozygous mice. Anim. Biotech. 4(1):89-107.

Hagemann, L.J. and **First, N.L.** 1992. Embryonic cytoplasmic extracts rescue murine androgenones to the blastocyst stage. Development 114(4): 997-1001

Takahashi, Y. and **First, N.L.** 1993. In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 39:561-567.

Eyestone, W.H., J.M. Jones and **First, N.L.** 1991. Oviduct-conditioned medium for the culture of early bovine embryos to the blastocyst stage. J. Reprod. Fertil. 92:59-64.

Eyestone, W.H. and **First, N.L.** 1991. Characterization of developmental arrest in early bovine embryos cultured in vitro. Theriogenology 35:613-624.

Hagen, D.R., R.S. Prather, M.M. Sims and **First, N.L.** 1991. Development of one-cell porcine embryos to the blastocyst stage in simple media. J. Anim. Sci. 69:1147-1150.

First, N.L. and R.S. Prather. 1991. Genomic potential in mammals. Differentiation 48:1-8.

Prather, R.S., M.M. Sims and **First, N.L.**. 1991. Culture of porcine embryos from one and two cells to blastocysts in sheep oviducts. Theriogenology 35:1147-1152.

Fulka, J., Jr., M.L. Leibfried-Rutledge and **First, N.L.** 1991. Effect of 6-dimethylaminopurine upon germinal vesicle breakdown of bovine oocytes. Mol. Reprod. Dev. 29:379-384.

Barnes, F.L. and **First, N.L.** 1991. Embryonic transcription in in vitro cultured bovine embryos. Molec. Reprod. Dev. 29:117-123.

Hagen, D.R., R.S. Prather and **First, N.L.** 1991. Response of porcine oocytes to electrical and chemical activation during maturation in vitro. Mol. Reprod. Dev. 28:70-73.

Saeki, K., M. Hoshi, M.L. Leibfried-Rutledge and **First, N.L.** 1991. In vitro fertilization and development of bovine oocytes matured in serum-free medium. Biol. Reprod. 44:256-260.

In re USSN 08/134,019
Declaration of Professor Janne (Attachment to Amendment filed Nov. 2, 1995)

COPY

DECLARATION IN CONNECTION WITH US 07/444,745 AND CORRESPONDING FOREIGN FILINGS BY DR. E.J. JÄNNE:

1. I am currently Professor of Biotechnology at the University of Kuopio (Finland) and hold several other academic positions including those of Director of the A.I. Virtanen Institute Univ. Kuopio and Vice-Chairman of the Department of Biochemistry and Biotechnology at the University of Kuopio. I am an editor and/or reviewer of over 30 scientific journals and am a co-author of over 200 scientific articles. I have been awarded with several awards and honors for my research work including, most recently, the Kuopio price of the city of Kuopio (in 1994) and the Matti Äyräpää price of the Finnish Medical Society Duodecim (in 1994). A copy of my CV is attached.

2. I have been asked by Gene Pharming Europe to read their patent application PRODUCTION OF RECOMBINANT POLYPEPTIDES BY BOVINE SPECIES AND TRANSGENIC METHODS and to comment on the state of the art of transgenic cattle technology at the priority date of this application (December 1, 1989). I have further been asked to discuss the considerations that led to my own research efforts in this field. Therefore I have read and reviewed the above application as it was filed at the US Patent and Trademark Office on December 1, 1989 [US 07/444,745]. I have further re-examined the background literature that is referenced in this declaration.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on Nov 2, 1995
By [signature]

3. I have been active in the field of transgenic animals since I became a professor of biotechnology at the University of Kuopio in 1988. Before my move to Kuopio my research interest was primarily focused on the metabolism and physiological function of polyamines. After I moved to Kuopio I expanded that interest by also studying physiological aspects of these molecules in transgenic mice and rats. I set up labs fitted to generating transgenic animals and have since generated many

different lines of transgenic mice and rats. During the establishment of my research group in Kuopio I decided that I should also form a group focused on production of valuable proteins (such as human erythropoetin) in milk of transgenic animals. I recruited my first Ph.D. student for this project in early 1990.

4. While contemplating the latter project and reading the relevant literature it became clear to me that several people had succeeded in developing gene constructs that were capable of directing the expression of foreign proteins to milk of mice. Of course, I realized that mice were not well-suited as production animals as their milk yield is too low. On the other hand, I also realized that the methods used for generating transgenic mice were impractical to generate cows (who would be the ideal milk producers) with a reasonable efficiency. Briefly, transgenic mice are generated in the following way: females are superovulated by hormonal treatment after which they are allowed to mate. Several hours after mating, the fertilized oocytes are removed from the oviducts of the females and are being injected with DNA. After injection the oocytes are implanted in the oviduct of a pseudo-pregnant female via a surgical procedure. It is unfeasible to use this procedure for the generation of transgenic cattle. An important reason herefore is that it would be prohibitively inefficient (and expensive!) to inject bovine oocytes obtained through superovulation as is done with mice. It would require large amounts of donor animals which would either have to undergo surgery or would have to be killed in which case the animals can not be used for human consumption anymore (due to the hormonal treatment). In addition, in contrast to mice, the number of oocytes per treated animal is low while it is also difficult and unpredictable to obtain oocytes from superovulated cattle that are in the right phase of the cell cycle for microinjection.

5. There had been reports in the literature that described attempts to generate transgenic cattle using, basically, the methods known for the generation of

transgenic mice. For instance Loskutoff et al. (Theriogenology (1986) 25:186) describe an effort to produce transgenic cattle. This abstract dramatically demonstrates the problems discussed in paragraph 4 of this declaration. A total of 75 surgical operations (51 to isolate oocytes and 24 to transfer the injected eggs) were needed to generate only three pregnancies. Given the fact that no follow-up paper was ever published, I assume that these pregnancies did not result in the birth of a transgenic calf. Biery et al. (Theriogenology (1988) 29: 224) published an abstract in which they described microinjection experiments in bovine oocytes obtained through superovulation. Although this abstract lacks experimental detail it is clear that large amounts of animals were used. Without showing scientific proof it was reported that three transgenic fetuses were generated. However, in only one tissue sample (derived from placental material) transgene expression was, reportedly, detected. It is, therefore, likely that only mosaic integration of the transgene was achieved; mosaic integration means that the DNA is integrated after the one cell-stage of the embryo, resulting in animals in which only some tissues contain the transgene. It is further relevant to note that there is no evidence in this abstract either that transgenic calves were actually generated. The above data, all based on oocytes obtained from superovulation (and fertilized in vivo), at the time indicated to me that alternative methods had to be developed in order to achieve a reasonable efficiency in the generation of transgenic cattle.

6. During the course of 1988 and 1989 several reports were published that described progress in culturing of bovine embryos in vitro (for instance Eyestone and First (1989) J. Reprod. Fert. 85:715-720; Bondioli PCT-patent application WO 89/07135; Gordon and Lu European Patent Application 89303741.6). However these references did not address the need to avoid methods to collect 'in vivo oocytes' (using superovulation). Rather, they described methods to culture bovine embryos in vitro after fertilization had occurred. Although this was and is a

relevant part of the entire protocol, I considered the source of oocytes as the real bottleneck of developing an efficient method of generating transgenic cattle.

7. Although protocols for in vitro maturation of bovine oocytes had been available for some time before I started contemplating a transgenic cattle project (in 1988 and 1989), it was my impression that the efficiencies that could be obtained were too low and too variable to be useful. For instance, Leibfield-Rutledge et al. (Theriogenology (1989) 31: 61-74) stated in a review (summarizing the state of the art in in vitro maturation and fertilization) that in vitro oocyte maturation would need much improvement before it could be used for domestic animal production and research (which were the applications that these authors were contemplating). At that time I believed that there was no reasonable expectation of success in using immature oocytes as a source for the generation of transgenic cattle. Certainly, the efficiencies as described by Gene Pharming Europe in their patent application, referred to in paragraph 2 of this declaration, were much higher than disclosed in the scientific literature at that time. It was not until 1990 that very high efficiencies of in vitro maturation were reported in the literature.

8. Moreover, I realized that the data available at the time of the filing of the Gene Pharming application, or indeed even after that filing date, did not address the effects that gene transfer protocols might have on the efficiencies of the entire in vitro process (i.e. the number of 'transgenic pregnancies' that could be reached by starting with a given number of immature oocytes). In fact, it was unpredictable how difficult or easy it would be to microinject in vitro matured oocytes as it could be expected that these cells would have different physiological properties compared to 'in vivo eggs'; such an experiment had, to my knowledge, never been tried in any species. For instance, it was quite conceivable at the time that the phasing of the cell cycle of 'in vitro oocytes' would be different from 'in vivo oocytes'. This would have had an impact on the visibility of the pronuclei and,

therefore, on the injection protocol. It was also quite conceivable at the time that the zona pellucida (the protein layer surrounding the oocyte) would have a different structure in 'in vitro oocytes' compared to 'in vivo oocytes'. Again, this would have impacted the injection protocol.

9. In early 1990 I became aware of a project being carried out by the University of Leiden in collaboration with researchers from Gene Pharming Europe and the Dutch Institute for Veterinary Research aimed at producing transgenic cattle by using immature oocytes as a source material. Apparently, these oocytes were matured and fertilized in vitro, with high efficiency, after which they were microinjected and further cultured in vitro. This integrated approach did increase the number of embryo's available for transfer significantly. The overall efficiency of the process, although low in absolute terms, was unexpectedly high. It was certainly surprising at the time that this approach immediately resulted in transgenic pregnancies and transgenic calves born.

10. It is now clear to me that the use of in vitro methods has additional advantages. First, the use of in vitro methods drastically reduces the burden on individual animals as no surgical steps have to be performed. This is very important from an animal-welfare point of view, but also relevant from an economic point of view since the costs are significantly reduced. Second, the use of immature oocytes makes it possible to highly synchronize the maturation and fertilization. Therefore, injection of these oocytes will become more reproducible. Third, it should be noted that even in the most efficient superovulation regimens a high proportion of non-transferable embryos resulting from fertilization failure and embryo degeneration, reduce the usefulness of that method in cases when high numbers of fertilized embryos are needed. Fourth, the use of an in vitro method does allow for manipulation of the embryo (such as morphological examination, biopsy taking etc.) at any stage of development.

11.Thus, within my department where expertise in molecular biology and transgenesis already existed, I formed a group of people building up expertise in the area of in vitro embryology and established a collaboration with an experimental animal research station. We started doing transfers of micro-injected (in vitro-cultured) bovine embryo's in 1991. Our first transgenic calf was born in 1993. This work has been published recently (Hyttinen et al. (1994) Bio/Technology 12: 606-608).

12.As is apparent from the publication referenced in section 11 and from various other publications from my department we have been able to successfully follow the method such as described in [US 07/444,745] without incurring significant difficulties. Although the frequency of transgene integration is still quite low, the efficiency of the in vitro methods will ensure success by routine repetitions of the same procedures. I believe that the availability of an integrated process for in vitro generation of bovine embryos <u>starting from immature oocytes</u> has been critical for our success. Not only did it allow for the generation of transgenic bovine embryos, we have also taken advantage of the fact that the in vitro process makes it possible to perform embryo manipulations: we have taken biopsies from embryos and analyzed those before transferring the remainder of the embryos to recipient animals.

13. As is clear from our own work, combined with recent research efforts from other groups, the in vitro technology as discussed above (and described in US 07/444,745) is unrelated to the transgene employed. It is clear that it has general applicability to generate any type of transgenic bovine.

Date:

~~September 13,~~ 1995

Signed:

Juhani Jänne